Inhibition of Acid Phosphatase Enzyme Activity in the Presence of Noncrystalline Calcium Phosphate and Nanocrystalline Calcium Apatite: a Preliminary Study

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Abstract

Background

Our bones are remodelled repeatedly during life. A new and "healthy" bone tissue replaces the old one. Accordingly, the bone degrading cells, the osteoclasts, prefer old and fatigued bone. The young bone mineral, i.e. calcium apatite, is less crystalline than the mature one. Is it possible that the osteoclasts distinguish between relatively old and new bone via its crystallinity?

Methodology/Principal Findings

Acid phosphatase is an enzyme largely expressed by the osteoclasts during resorption and therefore used as a marker of the cells activity. This study explores whether its enzymatic activity would be decreased in the presence of biomimitetically prepared noncrystalline calcium phosphate and nanocrystalline bone resembling calcium apatite. The results showed that both biomimetic samples decreased significantly the enzyme activity while synthetic calcium phosphate samples did not. Consistent with our hypothesis the noncrystalline calcium phosphate had the greater inhibition effect.

Conclusions/Significance

The *in vitro* data suggests that the bone-resorbing cells activity could be regulated by the degree of crystallinity of the bone mineral. These findings report that changes in the properties of biogenic inorganic materials induce specific interactions with biomolecules which in turn should define the cell behaviour. These results can establish an interesting mechanism of cell regulation if confirmed *in vivo*.

Introduction

Bone tissue is remodelled during our entire life. The old and fatigued bone is degraded by specialized cells called osteoclasts. Then a new bone is formed by another type of cells, the osteoblasts. The degradation itself consists of two parallel processes. The first one is the dissolution of the inorganic part of the bone, the mineral calcium apatite. The second one is the degradation of the organic, mainly collagenous, matrix by specific proteins and intracellular mechanisms.

Acid phosphatase (AcPho) is an osteoclast-produced enzyme, which is involved in the bone resorption process [1, 2]. As a member of the acid phosphatases family this enzyme deposphorylates proteins [3]. It is secreted by the human osteoclasts as a tartrate resistant acid phosphatase type 5b. In this form it is also involved in the generation of reactive oxygen species (ROS) used for the intracellular degradation of collagen fragments [4]. However, the role of this largely expressed during resorption enzyme and, therefore used as a marker of the osteoclast activity, is still not fully understood [5, 6]. It is known, however, that the most of the bone mineralization specific noncollagenous proteins are active only in phosphorylated state [7]. For instance, Ek-Rylander et al. showed [8] that once dephosphorylated bone sialoprotein and osteopontin could no longer promote the attachment of the osteoclasts to the bone surface.

Bone mineral matures with time and microcracks are generated [9]. There is a growing body of evidence that from mineralogical perspective the young and newly formed bone is generally disordered [10-16], if not fully amorphous, whereas the old bone is more crystalline, but still in nanometer dimensions [17-19]. It has been experimentally shown that the osteoclasts prefer to degrade old bone [20, 21]. Do the bone cells recognize the type of bone, i.e. young or old, via its crystallinity?

In this study we examined this question assuming that acid phosphatase activity (as a marker of bone resorption) should be influenced by the degree of crystallinity of the bone mineral calcium apatite. The enzymatic activity, i.e. the enzyme role in the degradation process, should be diminished in the presence of young disordered bone mineral. Therefore, we evaluated the change of the enzyme activity (EA) in the presence of biomimetically prepared noncrystalline calcium phosphate and nanosized apatite. These materials were to some extent similar in terms of crystallinity, chemical composition, surface area, etc. to the new and the old bone, respectively.

Experimental

Preparation of Materials

Noncrystalline or amorphous calcium phosphate (ACP) and nanocrystalline calcium apatite (CAp) were synthesized in simulated body fluid (SBF). SBF is a polyelectrolyte solution mimicking the composition of the human plasma [22] at physiological temperature (37 °C). The amorphous calcium phosphate was used as well as a precursor for the production of the apatite. The latter was achieved via maturation of the ACP in SBF for 24 h. For detailed description see Ref. [23].

The preparation method used in this study enables the production of bone-like noncrystalline calcium phosphate and nanocrystalline apatite. Both products are characterized with large surface area and carbonate content similar to the bone ones. The CAp contains very few or no hydroxyl groups in its structure, which is an important feature of bone apatite [24]. The samples are bone-resembling in terms of the concentrations of biogenic ions such as Mg^{2+}, Na^{+}, and K^{+} present in SBF [23].

As standards in this study were used commercially available hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HA) and dicalcium phosphate dihydrate ($CaHPO_4.2H_2O$, DCPD, mineral name: brushite). Both salts were purchased from Merck.

Instrumental Analysis

X-Ray Diffraction analyses were performed using Bruker D8 advanced diffractometer. The data obtained was used to calculate the particle size with TOPAS V.3 software and to compare the crystallinity of the samples. The phases were identified as well with FTIR by means of Nicolet Avatar 360 spectrometer and by the KBr pellet technique. Further analyses (SEM, surface area measurments, etc) of the bone-like salts (ACP and CAp) are reported elsewhere [23].

Enzyme Assay

Acid phosphatase from wheat germ (EC 3.1.3.2.), 0.4 U/mg activity, was purchased from Sigma. The substrate of the enzymatic reaction was freshly prepared 25 mM solution of 4-nitrophenylphosphate (Fluka) in phosphate buffer (pH 5.0). The reaction was stopped with the addition of 1M Na_2CO_3 (Merck). All assays were performed with deionized water and in sterile capped plastic tubes at 37°C.

The enzyme was mixed at a concentration of 100 μg/ml with 5 mg calcium phosphate in phosphate

buffer (pH 5). The total volume was 5 ml. A series of the above mixtures were incubated for 15 minutes in water bath with gentle agitation. At 15 min the substrate (25 mM solution of 4-nitrophenylphosphate) was added and the reaction was stopped after 3, 5, 9, 15, 20, and 30 minutes with 200 μL 1M Na_2CO_3. Finally, the absorbance of the product p-nitrophenolate was read at 405 nm. The sample/enzyme ratio was 20:1 to reflect as far as possible the natural conditions [3].

We tested additionaly whether the addition of the substrate 4-nitrophenylphosphate at time point 0 of the interaction between the calcium phosphate samples and the enzyme rather than in the 15th minute leads to difference in the final enzyme activity. No statistical difference was found between the two enzyme assays ($p = 0.369$, two-way ANOVA). It should be noted that we chose generally to measure the enzyme activity at this pH and buffer rather than the simulated body fluid. The reasons being that bone resorption is accomplished at pH 5.0 [1, 2], and about this pH is the optimum for the AcPho activity [25, 26]. However, at such an acidic pH some calcium phosphate dissolution occurs, but it is reasonable to presume that for the short time periods of the enzyme assay the effect is negligible [27].

Additional experiments were performed in SBF (pH = 7.4) as follows. Samples of all the studied calcium phosphate materials (1 mg/L in SBF) were added to 4.0 mL enzyme solution. Aliquots (200 μL) were taken at 0, 5, 10, 20, and 30 min and mixed with 1.0 mL acetate buffer, containing 50 μL 25 mM 4-nitrophenylphosphate. After 15 min incubation at 37 ºC, 500 μL 1M sodium carbonate was added to stop the reaction. The absorbance was read at 405 nm.

Protein Adsorption

The change of the enzyme concentration with time in the presence of the studied calcium phosphate materials was determined directly by spectrophotometry at 562 nm. Standard enzyme solution served as a control.

Statistical Analysis

The statistical analyses were performed by means of SPSS v.13.0. Analysis of variance (ANOVA) was used to evaluate the statistical significance of the data and the Tukey Post-hoc test to assess the variations of the enzymatic activity over time. Differences at $p > 0.05$ were considered statistically not significant for a sample size $n = 6$.

Results

XRD and FTIR Evaluation

The XRD spectra of the studied materials are shown in Figure 1. The HA and CAp spectra (Fig. 1, **a** and **b**, respectively) were similar since they have comparable particle size. Using the 002 reflections the evaluated crystallite sizes were about 7.73 nm for CAp and about 11.4 nm for HA. Thus both materials are nanosized calcium apatites. However, they differ chemically, since the CAp was synthesized in simulated body fluid and contained biogenic elements, while HA was relatively pure. CAp lacked hydroxyl groups almost entirely [23].

The ACP (Fig. 1, spectrum **c**) did not show distinct apatite diffraction peaks, and was thus considered as disordered, if not fully amorphous, material. In fact, the exact degree of "amorphousness" of the young bone mineral is not known with certainty [28, 29]. Therefore, a broad spectrum of non- or low crystalline calcium phosphates could be referred as "bone-like amorphous calcium phosphate". The ACP particle size could not be determined via XRD.

The DCPD diffractogram (ICDD file 02-0085) manifested a well-crystallized material (Fig. 1, **d**). Its crystallite size is greater than 1 micrometer.

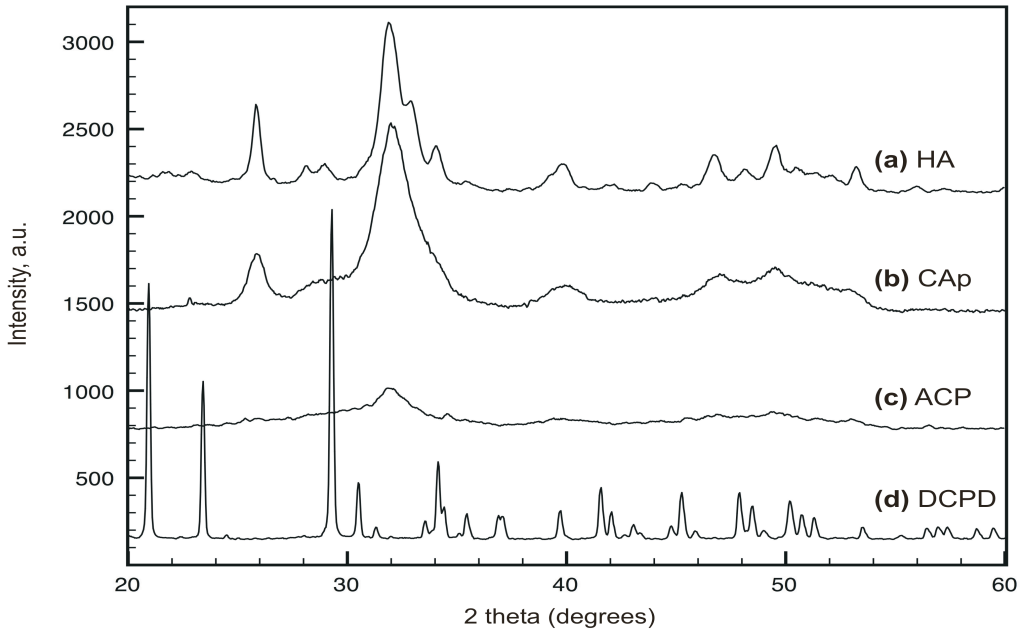

Figure 1. XRD sprectra of the samples. HA and CAp (spectra **a** and **b**) are characteristic for nanocrystalline calcium apatite. HA is more crystalline than CAp, but the difference is not great due to comparable crystallite size (see text). ACP (spectrum **c**) lacks distinct peaks and thus is generally deferred as amorphous or noncrystalline material. In contrast the brushite (diffractogram **d**) displays very well crystallized material with crystallite size larger than 1 micrometer.

The FTIR examination of the materials confirmed the XRD conclusions (Fig. 2). ACP lacked a resolved peak in the υ_4 phosphate vibrational region above 530-630 cm^{-1}(Fig. 2, **a**). This is typical for a disordered calcium phosphate phase [11, 12, 23, 30-32]. The spectra of CAp and HA (Fig. 2, **b** and **c**, respectively) were alike, which reflected their similarity in terms of crystallinity. CAp had high carbonate content than HA according to the relative intensity of the peaks at about 1450 and 873 cm^{-1}. The DCPD spectrum (Fig. 2, **d**) was characteristic for this calcium phosphate and confirmed its relatively high crystallinity.

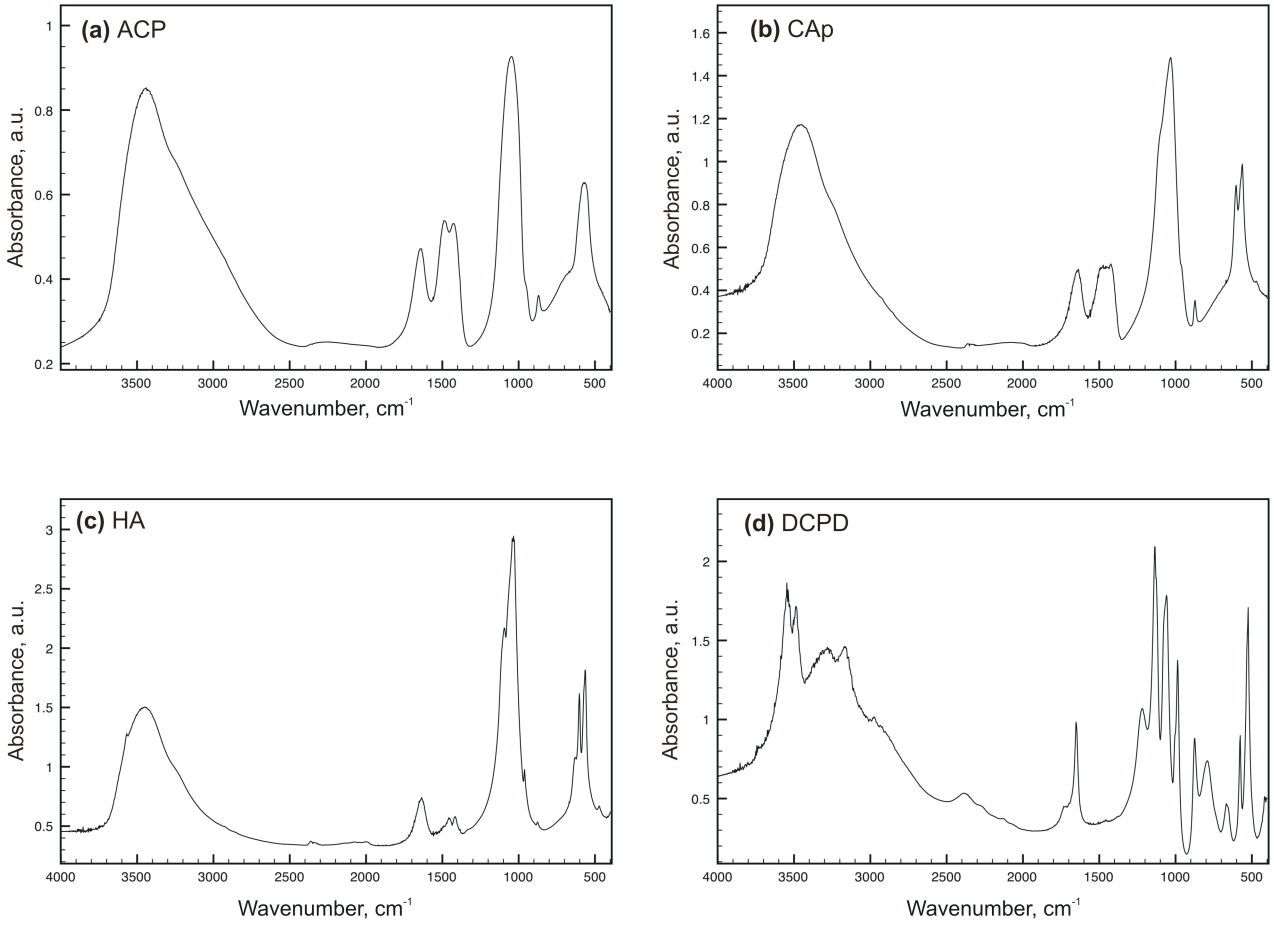

Figure 2. FTIR evaluation of the samples. ACP spectrum (**a**) manifests noncrystalline calcium phosphate, which typically lacks resolved band in the u_4 phosphate vibrational region about 530-630 cm^{-1}. The higher the crystallinity the greater the splitting in this region. This is well revealed in spectra **b** and **c** belonging to CAp and HA, respectively. Evidently, according to the FTIR analysis, HA is the more crystalline sample, which is consistent with the XRD data. DCPD spectrum (**d**) shows great splitting of the phosphate bands in all regions further confirming its greater crystallinity. The peaks about 1491 cm^{-1}, 1430 cm^{-1} and 873 cm^{-1} are due to carbonate substitution. Such substitution is generally found in bone mineral. The bands about 3400 cm^{-1} and 1640 cm^{-1} originate from bound water and/or hydroxyl ions in the apatite lattice.

Both ACP and CAp decrease the enzyme activity, but the ACP effect is stronger

The effects of ACP and CAp on the AcPho activity are shown in Figure 3. Both materials decreased the AcPho activity, with the ACP being the stronger inhibitor. The disordered calcium phosphate phase, i. e. the ACP, inhibited the enzyme activity by 9.4% more than CAp in the 30th minute ($p<0.001$). This result is consistent with our initial hypothesis.

It should be noted that in general before the fifth minute the measurements (in all studied samples) were not very well duplicated. The enzyme activity difference in the presence of ACP at the 3rd and 5th minute is not statistically significant ($p = 0.19$).

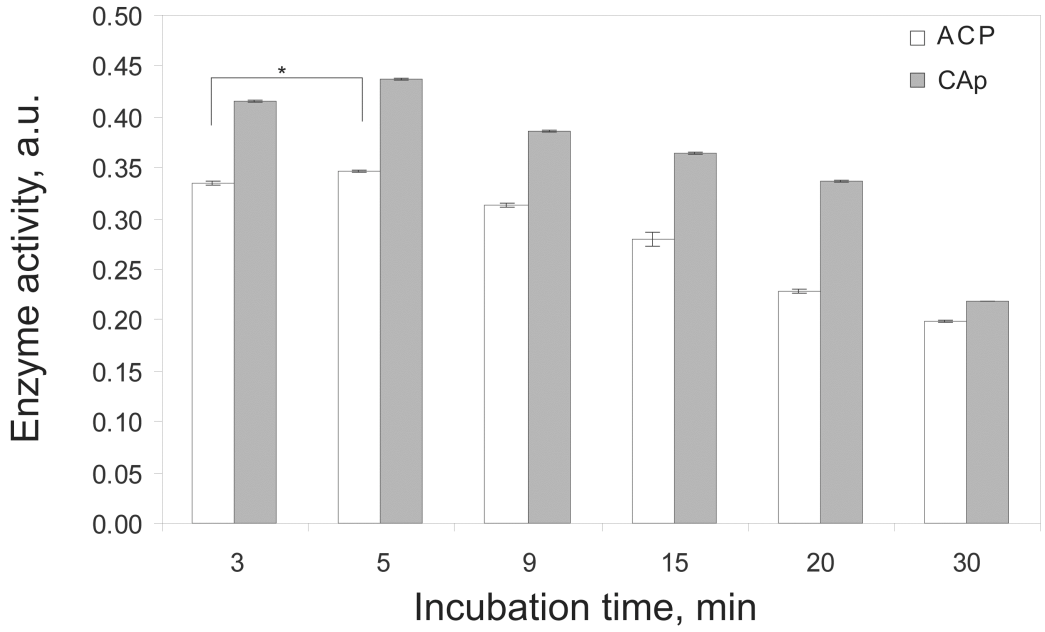

Figure 3. Changes in acid phosphatase enzyme activity in presence of ACP (White columns) and CAp (Grey columns). Error bars represent standard error of the mean [33]. Both samples decrease the activity of the enzyme ($P < 0.001$ for CAp and $p = 0.001$ for ACP) after the fifth minute, however the inhibition effect of ACP is greater ($p < 0.001$). Asterisk symbol denotes not statistically significant difference between samples (see text).

HA and DCPD do not decrease the enzyme activity

Surprisingly, both brushite and HA did not influence the AcPho activity. After 5 minutes incubation of the samples the mean values of the enzyme activity remained the same until the end of the assay ($EA_{(DCPD)} = 0.534$; $SD = 0.003$ and $EA_{(HA)} = 0.524$; $SD = 0.008$). This observation suggests that the chemical nature of the samples is not responsible for the observed EA decrease. Moreover, we can

exclude the crystallite size as a reason for the EA inhibition since CAp and HA have very similar crystallite dimensions.

SBF and Ca^{2+} ions do not decrease the AcPho activity

To test whether the biogenic ions (Na^+, K^+, Mg^{2+}, Cl^-, CO_3^{2-}) present in ACP and CAp were responsible for the observed phenomena, the experiment was performed in SBF. If true, the simulated body fluid itself would lower the activity. However, the results obtained in SBF with or without samples kept the trend of the ones in phosphate buffer. The SBF pH of 7.4 did not have significant influence on the enzyme activity (data not shown).

We hypothesized as well that the enzyme activity was decreased by calcium ions released from the samples during the assay. This idea is highly unlikely [25, 34], but reasonable since ACP has both the greater solubility and the stronger inhibition effect on the EA compared to CAp. However, we found no correlation between the calcium concentrations in both SBF and phosphate buffer and the AcPho activity (data not shown).

The enzyme activity decrease is not due to protein adsorption

We tested whether a protein adsorption process caused the observed trends. The results are shown on Table 1. They indicated surprisingly that there was no enzyme adsorption by any of the studied materials. Thus, we must rule out the hypothesis that an adsorption is responsible for the inhibition of the acid phosphatase enzyme activity in the presence of bone-like minerals.

Table 1. Adsorption of acid phosphatase in the presence of the studied samples.

Substance	Absorbance @ 562 nm [mean value ± SD]			
	Control	5 min	15 min	30 min
ACP	1.295 ± 0.001	1.297 ± 0.003	1.295 ± 0.002	1.307 ± 0.003
CAp	1.218 ± 0.001	1.217 ± 0.000	1.225 ± 0.000	1.227 ± 0.003
DCPD	1.143 ± 0.003	1.147 ± 0.003	1.144 ± 0.002	1.148 ± 0.002
HA	1.292 ± 0.002	1.292 ± 0.003	1.295 ± 0.002	1.295 ± 0.001

The data is reported as a mean value of four replicates with their standard deviations (SD). Some time points are omitted from the Table since there is no statistical significant difference with the ones shown.

Discussion

This study illustrates that inorganic materials can interact specifically with an enzyme, changing its enzymatic activity. An intriguing hypothesis can be proposed. If the results are confirmed under more native conditions, this will show that particular inorganic material-biomolecule interaction can command the cell behaviour. Briefly, we speculate that the osteoclasts resorption activity, which is greater in the case of the old and more crystalline bone, is somehow regulated via the inhibition of the acid phosphatase enzyme. The latter protein is strongly expressed *in vivo* during resorption and therefore is used as a marker of the osteoclastic activity. However the exact role(s) of the enzyme in the degradation process is still unknown.

What causes the observed inhibition of the enzyme activity in the presence of bone-like minerals, but not in the presence of other calcium phosphates salts? Why does the noncrystalline biomimetic precursor show greater inhibition effect than the bone-like nanocrystalline apatite?

As reported here it is highly unlikely that these findings can be ascribed to chemical nature of the samples, adsorption, or even crystallinity since CAp and HA have almost the same crystallite characteristics. Thus, we believe that some kind of surface phenomenon is driving the observed trends. One possibility is that the surface charge of amorphous calcium phosphate is positive [35], while the one of calcium apatite is negative. The greater the crystallinity of the latter, the greater its negative surface charge [36]. However, to the best of our knowledge, the isoelectric point (pI) of wheat germ AcPho is not exactly defined since the enzyme is constituted by several isozymes with pI values ranging from about 4.0 to 7.5 [37, 38]. Thus, it is impossible to define the AcPho surface charge in phosphate buffer (pH = 5) and to judge with certainty whether the particles electric charge is responsible for the observed phenomenon.

There are other difference between the studied materials, which should be taken into consideration, such as the following: ACP and CAp have larger surface areas than HA and DCPD (ACP has the largest of all); CAp does not contain hydroxyl ions in its crystal lattice in contrast to HA [23]; Some transformation, i.e. crystallisation, occurs with the ACP sample during the time of the enzyme activity essay [39, 40]; Both ACP and CAp have high carbonate content (5-7 wt%) similar to bone mineral. HA is almost carbonate free (Fig. 2, **c**); DCPD has different type crystal lattice than HA and CAp. Hopefully, some of these could help in finding the explanation of the observed phenomenon in the future. One should assume as well that the human osteoclats AcPho is tartrate resistant and contains two iron ions (II and III) [3]. The wheat germ enzyme used here is not tartrate

resistant and does not contain Fe ions as we confirmed by means of atomic adsorption spectroscopy.

In conclusion, this study indicates the possibility that according to its crystallinity the bone mineral itself control the osteoclast acid phosphatase activity and hence probably the cell behaviour. In compliance we found that both noncrystalline calcium phosphate and nanocrystaline apatite inhibit the enzyme activity, with the greater effect manifested by the amorphous polymorph.

References

[1] H.K. Vaananen, Y.K. Liu, P. Lehenkari, T. Uemara, How do osteoclasts resorb bone?, Materials Science & Engineering C-Biomimetic and Supramolecular Systems 6 (1998) 205-209.

[2] H.K. Vaananen, H. Zhao, M. Mulari, J.M. Halleen, The cell biology of osteoclast function, Journal of Cell Science 113 (2000) 377-381.

[3] H. Bull, P.G. Murray, D. Thomas, A.M. Fraser, P.N. Nelson, Acid phosphatases, Journal of Clinical Pathology-Molecular Pathology 55 (2002) 65-72.

[4] J.M. Halleen, S. Raisanen, J.J. Salo, S.V. Reddy, G.D. Roodman, T.A. Hentunen, P.P. Lehenkari, H. Kaija, P. Vihko, H.K. Vaananen, Intracellular fragmentation of bone resorption products by reactive oxygen species generated by osteoclastic tartrate-resistant acid phosphatase, Journal of Biological Chemistry 274 (1999) 22907-22910.

[5] A.R. Hayman, Tartrate-resistant acid phosphatase (TRAP) and the osteoclast/immune cell dichotomy, Autoimmunity 41 (2008) 218-223.

[6] J.M. Halleen, S.L. Tiitinen, H. Ylipahkala, K.M. Fagerlund, H.K. Vaananen, Tartrate-resistant acid phosphatase 5b (TRACP 5b) as a marker of bone resorption, Clinical Laboratory 52 (2006) 499-509.

[7] A. George, A. Veis, Phosphorylated Proteins and Control over Apatite Nucleation, Crystal Growth, and Inhibition, Chemical Reviews 108 (2008) 4670-4693.

[8] B. Ek-Rylander, M. Flores, M. Wendel, D. Heinegard, G. Andersson, Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion in vitro., Journal of Biological Chemistry 269 (1994) 14853-14856.

[9] D. Taylor, J.G. Hazenberg, T.C. Lee, Living with cracks: Damage and repair in human bone, Nature Materials 6 (2007) 263-268.

[10] M.J. Olszta, X.G. Cheng, S.S. Jee, R. Kumar, Y.Y. Kim, M.J. Kaufman, E.P. Douglas, L.B. Gower, Bone structure and formation: A new perspective, Mater. Sci. Eng. R-Rep. 58 (2007) 77-116.

[11] J. Mahamid, A. Sharir, L. Addadi, S. Weiner, Amorphous calcium phosphate is a major component of the forming fin bones of zebrafish: Indications for an amorphous precursor phase, Proceedings of the National Academy of Sciences of the United States of America 105 (2008) 12748-12753.

[12] J.D. Termine, A.S. Posner, Infrared Analysis of Rat Bone - Age Dependency of Amorphous and Crystalline Mineral Fractions, Science 153 (1966) 1523-&.

[13] N.J. Crane, V. Popescu, M.D. Morris, P. Steenhuis, M.A. Ignelzi, Raman spectroscopic

evidence for octacalcium phosphate and other transient mineral species deposited during intramembranous mineralization, Bone 39 (2006) 434-442.

[14] S. Gajjeraman, K. Narayanan, J.J. Hao, C.L. Qin, A. George, Matrix macromolecules in hard tissues control the nucleation and hierarchical assembly of hydroxyapatite, J Biol Chem 282 (2007) 1193-1204.

[15] L.N.Y. Wu, G.R. Sauer, B.R. Genge, W.B. Valhmu, R.E. Wuthier, Effects of analogues of inorganic phosphate and sodium ion on mineralization of matrix vesicles isolated from growth plate cartilage of normal rapidly growing chickens, Journal of Inorganic Biochemistry 94 (2003) 221-235.

[16] J. Tao, H. Pan, Y. Zeng, X. Xu, R. Tang, Roles of amorphous calcium phosphate and biological additives in the assembly of hydroxyapatite nanoparticles, J Phys Chem B 111 (2007) 13410-13418.

[17] A. Bigi, G. Cojazzi, S. Panzavolta, A. Ripamonti, N. Roveri, M. Romanello, K.N. Suarez, L. Moro, Chemical and structural characterization of the mineral phase from cortical and trabecular bone, J Inorg Biochem 68 (1997) 45-51.

[18] S.V. Dorozhkin, M. Epple, Biological and medical significance of calcium phosphates, Angew Chem Int Ed 41 (2002) 3130-3146.

[19] F. Peters, K. Schwarz, M. Epple, The structure of bone studied with synchrotron X-ray diffraction, X-ray absorption spectroscopy and thermal analysis, Thermochim Acta 361 (2000) 131-138.

[20] K. Henriksen, D.J. Leeming, I. Byrjalsen, R.H. Nielsen, M.G. Sorensen, M.H. Dziegiel, T.J. Martin, C. Christiansen, P. Qvist, M.A. Karsdal, Osteoclasts prefer aged bone, Osteoporosis International 18 (2007) 751-759.

[21] M.A. Karsdal, I. Byrjalsen, D.J. Leeming, M. Sorensen, R. Nielsen, C. Christiansen, K. Henriksen, P. Qvist, Old bones support osteoclastogenesis superior to that of young bone, resulting in preferential resorption of old bone, Osteoporosis International 17 (2006) S134-S134.

[22] A.C. Tas, Synthesis of biomimetic Ca-hydroxyapatite powders at 37 degrees C in synthetic body fluids, Biomaterials 21 (2000) 1429-1438.

[23] Ya. Pekounov, O.E. Petrov, Bone resembling apatite by amorphous-to-crystalline transition driven self-organisation, Journal of Materials Science-Materials in Medicine 19 (2008) 753-759.

[24] J.D. Pasteris, B. Wopenka, J.J. Freeman, K. Rogers, E. Valsami-Jones, J.A.M. van der Houwen, M.J. Silva, Lack of OH in nanocrystalline apatite as a function of degree of atomic order: implications for bone and biomaterials, Biomaterials 25 (2004) 229-238.

[25] B.K. Joyce, S. Grisolia, Purification and prpperties of a nonspecific acid phosphatase from wheat germ, Journal of Biological Chemistry 235 (1960) 2278-2281.

[26] Y. Kawarasaki, H. Nakano, T. Yamane, Purification and some properties of wheat germ acid phosphatases, Plant Science 119 (1996) 67-77.

[27] K. Onuma, A. Ito, Cluster growth model for hydroxyapatite, Chem Mater 10 (1998) 3346-3351.

[28] M.D. Grynpas, Transient precursor strategy or very small biological apatite crystals?, Bone 41 (2007) 162-164.

[29] S. Weiner, Transient precursor strategy in mineral formation of bone, Bone 39 (2006) 431-433.

[30] S.J. Gadaleta, E.P. Paschalis, F. Betts, R. Mendelsohn, A.L. Boskey, Fourier transform infrared spectroscopy of the solution-mediated conversion of amorphous calcium phosphate to hydroxyapatite: New correlations between X-ray diffraction and infrared data, Calcified Tissue International 58 (1996) 9-16.

[31] Y.B. Li, T. Wiliana, K.C. Tam, Synthesis of amorphous calcium phosphate using various types of cyclodextrins, Mater Res Bull 42 (2007) 820-827.

[32] S. Somrani, C. Rey, M. Jemal, Thermal evolution of amorphous tricalcium phosphate, J Mater Chem 13 (2003) 888-892.

[33] G. Cumming, F. Fidler, D.L. Vaux, Error bars in experimental biology, J. Cell Biol. 177 (2007) 7-11.

[34] S.C. Tso, Y.R. Chen, Isolation and characterization of a group III isozyme of acid phosphatase from rice plants, Botanical Bulletin of Academia Sinica 38 (1997) 245-250.

[35] P.B.Y. Ofir, R. Govrin-Lippman, N. Garti, H. Furedi-Milhofer, The influence of polyelectrolytes on the formation and phase transformation of amorphous calcium phosphate, Cryst Growth Des 4 (2004) 177-183.

[36] H.M. Kim, T. Himeno, T. Kokubo, T. Nakamura, Process and kinetics of bonelike apatite formation on sintered hydroxyapatite in a simulated body fluid, Biomaterials 26 (2005) 4366-4373.

[37] R. Hooley, Gibberellic acid controls specific acid pshosphatase isozymes in aleurone cells and protoplasts of Avena Fatua L, Planta 161 (1984) 355-360.

[38] T. Akiyama, S. Yamamoto, Immunochemical differences in acid phosphatase isozymes from wheat germ, Agricultural and Biological Chemistry 50 (1986) 437-440.

[39] A.L. Boskey, A.S. Posner, Conversion of Amorphous Calcium Phosphate to Microcrystalline Hydroxyapatite - pH-Dependent, Solution-Mediated, Solid-Solid Conversion, J Phys Chem 77 (1973) 2313-2317.

[40] N.C. Blumenthal, J.M. Holmes, A.S. Posner, Effect of Preparation Conditions on Properties and Transformation of Amorphous Calcium Phosphate, Mater Res Bull 7 (1972) 1181-1190.

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